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RFamide-related peptide 3 and gonadotropin-releasing hormone-II are autocrine–paracrine regulators of testicular function in the boar

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Widespread use of artificial insemination in swine requires millions of doses of boar semen each year. Subfertility of boars remains a major constraint, which can impact the reproductive efficiency of thousands of sows, so a better understanding of testicular function is needed in order to develop methods to improve semen production. With this in mind, the effects of RFamide-related peptide 3 (RFRP3) and Gonadotropin-releasing hormone-II (GnRH-II) on gonadotropin secretion and testicular function of pigs are reviewed here. Receptors for RFRP3 are present in the pig hypothalamus, adenohypophysis, and testis. Evidence from in vitro studies indicates that RFRP3 could be a hypophysiotropic hormone in the pig by suppressing secretion of GnRH-I from the hypothalamus and luteinizing hormone (LH) from the pituitary gland; however, effects of RFRP3 on in vivo secretion of LH in pigs are minimal. Within the pig testis, RFRP3 suppresses testosterone secretion by inhibiting steroidogenic enzymes. GnRH-II and its receptor (GnRHR-II) are abundant in pig testes. Interstitial cells express GnRHR-II, and exogenous GnRH-II robustly stimulates secretion of testosterone in boars, despite minimal secretion of LH. Data illustrate that GnRH-II directly stimulates secretion of testosterone from the testes of mature boars. Thus, the primary function of RFRP3 and GnRH-II in the boar appears to be autocrine–paracrine inhibition and stimulation, respectively, of testosterone secretion within the testis. A better understanding of changes in the RFRP3 and GnRH-II systems within the testis during development will provide important clues about how to improve the testicular function of boars.

KEYWORDS
GnRH-II, pig, RFamide peptides, testes, testosterone

How RFamide-related peptides and gonadotropin-releasing hormone II interact during development will be important in order to provide clues to the pubertal transition of testosterone secretion in the boar.

Abbreviations: FSH, Follicle-stimulating hormone; GnIH, Gonadotropin-inhibitory hormone; GnRH[R], Gonadotropin-releasing hormone [receptor]; hCG, Human chorionic gonadotropin; LH, Luteinizing hormone; NPFF[R], Neuropeptide FF [receptor]; *NPVF*, Neuropeptide VF; PKA, Protein kinase A; QRFP, pyroglutamylated RFamide peptide; RFRP, RFamide-related peptide.

1 | INTRODUCTION

Reproductive failure is a major economic burden for the swine industry (Lopes, Sanchez-Osorio, Bolarin, Martinez, & Roca, 2014). Often overlooked, boar subfertility impacts reproductive outcome by reducing conception rate and litter size (Swierstra & Dyck, 1976). Over 11.6 million sows were farrowed in the United States in 2015 (USDA, 2015), with >90% of them inseminated artificially (Flowers, 2015). The wide-spread use of artificial insemination in the swine

industry requires over 30 million doses of semen each year (Kuster & Althouse, 2008). Consequently, the impact of a single sub-fertile boar can affect thousands of females (Foxcroft, Dyck, Ruiz-Sanchez, Novak, & Dixon, 2008), so artificial insemination doses of boar semen are packaged as pooled collections from multiple sires to achieve successful pregnancy rates in the field. The population of sub-fertile boars is growing, however, and conventional semen analysis cannot identify these individuals (Dyck et al., 2011). Thus, a better understanding of testicular function is needed in order to develop methods that improve semen production and quality in the pig.

The hypothalamic–pituitary–gonadal axis regulates testis function via classic endocrine mechanisms. Binding of Gonadotropin-releasing hormone (GnRH-I) to its receptor (GnRHR-I) within the anterior pituitary gland stimulates the synthesis and secretion of the gonadotropins Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH). These gonadotropins travel through the blood to the testis, where they bind to their cognate receptors. FSH promotes spermatogenesis within the seminiferous tubules, whereas LH stimulates the synthesis and secretion of the prominent androgen, testosterone, by Leydig cells. Testosterone is required for male reproductive success (e.g., spermatogenesis, accessory sex gland function, and libido).

Negative feedback of gonadal steroids and inhibin at the hypothalamus and anterior pituitary gland was thought to be the primary regulator of gonadotropin secretion in pigs (Ford, Klindt, & Wise, 2001). Neuropeptides and their receptors are also expressed in the gonad, and can directly affect gonadal function (Iwashita & Catt, 1985; Jegou, Brekke, Naess, Torjesen, & Hansson, 1985; Kakar, Musgrove, Devor, Sellers, & Neill, 1992; Nikula & Huhtaniemi, 1988; Petersson, Emons, & Hammar, 1989); however, information indicating that neuropeptides may act as paracrine regulators of testicular function in the boar is lacking. Here, we review and present evidence that RFamide peptides, the products of the *NPVF* gene, are not major hypophysiotropic hormones in the pig, but could be important paracrine factors in the negative regulation of testosterone secretion in boars. Likewise, we review recent evidence from our laboratories that suggest a role for a second isoform of GnRH (GnRH-II) and its receptor in the positive regulation of steroidogenesis and spermatogenesis in the boar testis.

2 | RFamide PEPTIDES

RFamide peptides are structurally related neuropeptides that regulate a number of biological functions, including nociception, food intake, and reproduction (Fukusumi, Fujii, & Hinuma, 2006; Tsutsui & Ukena, 2006; Yang, Tao, & Iadarola, 2008). The five families of RFamide peptides are Prolactin-releasing peptides (PrRP), pyroglutamylated RFamide peptides (QRFP), Kisspeptins, Neuropeptide FF (NPFF), and peptide products of the *NPVF* gene, called RFamide-related peptides (RFRP) (Fukusumi et al., 2006; Tsutsui et al., 2010; Ukena, Vaudry, Leprince, & Tsutsui, 2011). The RFamide peptides are characterized by an amidated C-terminal amino acid motif that is essential for receptor binding and activation (Yin, Ukena, Ubuka, & Tsutsui, 2005). The C-terminal amino acid motif of the RFRP peptides is LPXRF, where X can be either leucine (L) or glutamine (Q) (Ukena, Iwakoshi, Minakata, & Tsutsui, 2002; Ukena & Tsutsui, 2005). The porcine *NPVF* gene precursor protein can be cleaved to produce three RFRP fragments (RFRP1, -2, and -3) (Li et al., 2012; Thorson et al., 2017). The biological function of these RFRP peptides in the pig have yet to be fully explored, although RFRP3 is considered the mammalian ortholog to avian Gonadotropin-inhibitory hormone (GnIH) (Clarke et al., 2008; Kriegsfeld et al., 2006; Murakami et al., 2008), which inhibits the hypothalamic–pituitary–gonadal axis of birds (Tsutsui et al., 2007; Tsutsui, Ubuka, Bentley, & Kriegsfeld, 2012).

Understanding how these neuropeptides might regulate reproduction in pigs requires knowledge of what receptors they might work through. The NPFF peptides are reported to bind NPFF receptor 2 (NPFFR2, also called GPR74) (Bonini et al., 2000; Liu et al., 2001). The RFRPs, however, preferentially bind NPFFR1 (also called GPR147) although these peptides can also bind and activate NPFFR2 at a lower affinity (Hinuma et al., 2000). This bimodality is important because *NPFFR2* was identified as a candidate gene for puberty in pigs (Nonneman et al., 2016). Indeed, *NPFFR1* and *NPFFR2* transcripts are present in the hypothalamus, adenohypophyses, and the gonad of the pig (Li et al., 2012; Thorson et al., 2017), and are altered with developmental state (Thorson et al., 2017). Thus, RFRPs may affect reproduction in the pig at the level of the hypothalamic-pituitary axis or directly at the gonad—although the considerable promiscuity among NPFF receptors for RFamide peptides should also be considered. Kisspeptin, for example, can bind and activate NPFF receptors (Lyubimov et al., 2010; Oishi et al., 2011). Conversely, a specific NPFF receptor antagonist, RF9 (adamantanecarbonyl-Arg-Phe-NH_4), was found to bind and activate the Kisspeptin receptor (Min et al., 2015; Simonin et al., 2006). Such lack of strict receptor specificity has made it difficult to conclusively determine the mechanisms through which many of the RFamide peptides affect reproduction in pigs.

2.1 | RFamide peptides and gonadotropin secretion

Kisspeptin is well-characterized for its potent stimulation of gonadotropin secretion in many species, including pigs (Lents, Heidorn, Barb, & Ford, 2008). This neuropeptide acts at the hypothalamus (Arreguin-Arevalo, Lents, Farmerie, Nett, & Clay, 2007), where it directly stimulates GnRH-I neurons that express Kisspeptin receptor (Messager et al., 2005). The QRFP peptides, 43RFa and 26RFa, are primarily involved in regulating food intake (Lectez et al., 2009; Ukena et al., 2011). Navarro and colleagues showed that the pituitary gland of rats expressed QRFP receptors, and that QRFP peptides stimulated basal secretion of LH and potentiated the GnRH-I-stimulated release of LH from primary cultures of pituitary cells from rats (Navarro et al., 2006). When 26RFa was administered centrally and peripherally, it stimulated secretion of LH in female but not male rats (Navarro et al., 2006). Our laboratory has treated pigs with 26RFa and discovered that it stimulates secretion of growth hormone but did not influence secretion of LH (Lents, unpublished), suggesting that QRFPs do not regulate

gonadotropin secretion in pigs. We, therefore, sought to gain greater understanding of RFRPs because of their potential homology to avian GnIH.

Avian GnIH was discovered, in 2000, as a hypothalamic RFamide peptide (Tsutsui et al., 2000), and was so named because it suppressed the reproductive neurosecretory axis of birds through inhibition of LH secretion at the level of the hypothalamus and anterior pituitary gland (Bentley et al., 2006; Osugi et al., 2004; Ubuka, Ukena, Sharp, Bentley, & Tsutsui, 2006). RFRP3 is generally regarded as the mammalian homolog to avian GnIH peptide because it shares the same C-terminal amino acid motif (Kriegsfeld et al., 2006). The RFRP peptides were identified in the hypothalamus of all mammals studied to date, and are expressed in the paraventricular and dorsomedial hypothalamic areas (Johnson, Tsutsui, & Fraley, 2007; Kriegsfeld et al., 2006; Thorson et al., 2014). RFRP neurons in these areas project fibers to a number of hypothalamic regions, including those containing GnRH-I neurons (Clarke et al., 2008; Qi, Oldfield, & Clarke, 2009; Ubuka et al., 2009). Many GnRH-I neurons lie in close apposition to RFRP fibers (Bentley, Perfito, Ukena, Tsutsui, & Wingfield, 2003; Johnson et al., 2007; Kriegsfeld et al., 2006; Smith et al., 2008), with a subpopulation of GnRH-I neurons expressing *NPFFR1* (Rizwan et al., 2012; Ubuka et al., 2008, 2012). Expression of *NPFFR1* was also noted in GnRH-II neurons in starlings (Ubuka et al., 2008). This anatomical organization suggests that RFRPs can alter GnRH-I secretion. Indeed, RFRP3 inhibited firing of GnRH-I neurons in hypothalamic slices from rats and mice (Anderson, Relf, Rizwan, & Evans, 2009; Ducret, Anderson, & Herbison, 2009; Wu, Dumalska, Morozova, van den Pol, & Alreja, 2009). Secretion of GnRH-I from hypothalamic tissue of pigs was also attenuated by RFRP3 in vitro (Li et al., 2013), so it is possible that RFRP3 could inhibit gonadotropin secretion in the pig by acting in the hypothalamus to suppress secretion of GnRH-I. Interestingly, transcription of the *NPVF* gene (RFRP precursor) in the dorsomedial nucleus of mice decreased with sexual maturity (Poling, Kim, Dhamija, & Kauffman, 2012). *NPFFR1* and *NPFFR2* mRNA are also expressed in the hypothalamus of the pig, and their expression is altered with sexual maturation (Thorson et al., 2017). This raises the possibility that pre-pubertal inhibition in GnRH-I secretion in pigs may be partly mediated by RFRP3.

In early studies using anterior pituitary cells from sheep and cattle, RFRP3 was shown to suppress LH secretion directly from gonadotrope cells (Clarke et al., 2008; Kadokawa et al., 2009), which express *NPFFR1* (Smith, Young, Veldhuis, & Clarke, 2012). The anterior pituitary gland of the pig expresses *NPFFR1* and *NPFFR2* (Li et al., 2012), and their expression in gilts is dramatically decreased with the onset of puberty, suggesting that the onset of puberty in the pig is concomitant with reduced inhibition of LH secretion in the adenohypophesis by RFRP. Consistent with this hypothesis, Li and colleagues demonstrated that RFRP3 suppressed GnRH-I-stimulated secretion of LH from primary cultures of porcine pituitary cells (Li et al., 2013).

Strong neuroanatomical and in vitro evidence support a role for RFRP3 in regulating LH secretion in the pig. Results from in vivo studies of other mammals, such as rodents, however, have been decidedly equivocal (Ancel et al., 2012; Caraty et al., 2012; Pineda et al., 2010; Rizwan, Porteous, Herbison, & Anderson, 2009). Differences in experimental approach and physiological states of animal models may explain some of these incongruences, but even at the same dose in the same species, RFRP3 does not always have consistent effects on secretion of LH. For example, RFRP3 was first reported to suppress the number of LH pulses in ovariectomized ewes (Clarke et al., 2008), whereas others later found that RFRP3 did not regulate pulsatile secretion of LH in ovariectomized ewes (Decourt et al., 2016). Similarly, Thorson and colleagues demonstrated in that the cellular machinery associated with RFRP3 function is present in the mare hypothalamus and adenohypophesis, but RFRP3 did not affect pulsatile secretion of LH (Thorson et al., 2014).

We conducted the first in vivo study on the effects of RFRP3 on LH secretion in pigs using ovariectomized gilts (Thorson et al., 2017). Gilts were treated with RFRP3 either centrally, in the lateral ventricles, or peripherally, in the jugular vein. No effect of RFRP3 on pulsatile secretion of LH was observed, so we speculated that the presence of gonadal steroids may be necessary for RFRP3 to have a suppressive effect on LH secretion. We tested our hypothesis by treating mature Chinese Meishan boars with RFRP3 (Thorson et al., 2015). Chinese Meishan boars were used because they have greater concentrations of LH and gonadal steroids than commercial white composite boars (Wise, Lunstra, & Ford, 1996; Zanella, Ford, Wise, & Hamernik, 1996), so we reasoned that the suppressive effects of RFRP3 on LH secretion would be more evident in this breed. When Chinese Meishan boars were treated with a pharmacological dose of RFRP3 in the jugular vein, pulsatile secretion of LH was inhibited in some boars. Not all boars responded equally, though: some boars showed a delayed response whereas others exhibited only a minimal change in LH. The delayed response is similar to what we observed for ovariectomized gilts given a similarly large dose of RFRP3 (Thorson et al., 2017). The basis for such varied response to RFRP3 among individual boars is not immediately evident, but could be related to differences in NPFF receptors abundance. Overall, these data suggest that RFRP3 is not a major hypophysiotropic hormone in the pig.

2.2 | RFamide peptides and paracrine regulation of testis function

Although RFRP and NPFF receptors are most abundant in the hypothalamus and adenohypophysis, they are expressed in other tissues including the gonad of pigs (Li et al., 2012). The testes of boars also possess mRNA for *NPFFR1* and *NPFFR2*, and expression of the *NPVF* gene (RFRP precursor) was observed in interstitial cells (Zheng et al., 2015). This expression profile is analogous to that of avian GnIH and its receptor, which are expressed and localized in the interstitium of avian testes (Bentley et al., 2008; McGuire & Bentley, 2010). Treating testis cultures from house sparrows with GnIH suppressed gonadotropin-stimulated secretion of testosterone (McGuire & Bentley, 2010). Together, these observations raise the possibility that RFRP peptides have a paracrine effect on boar testis function, so we used testicular explant cultures to test the hypothesis that RFRP3 inhibits testosterone release. Basal release of testosterone was not affected by RFRP3, but RFRP3 did inhibit the human Chorionic

gonadotropin (hCG)-stimulated secretion of testosterone by 3 hr after exposure ($p < 0.01$) (Figure 1). Secretion of testosterone from primary cultures of Leydig cells from Chinese Meishan boars was similarly inhibited when treated with RFRP3 (Zheng et al., 2015). The RFRP3-induced inhibition of testosterone secretion from testis cultures of boars resulted from reduced expression of LH receptor, Steroidogenic acute regulatory protein, Cytochrome P450 11A1, and 3β-hydroxysteroid dehydrogenase (Zheng et al., 2015), which is similar to that observed for mice (Anjum, Krishna, & Tsutsui, 2014). In the LβT2 murine gonadotrope cell line, RFRP3 blocked GnRH stimulation of *Lhβ* gene transcription by inhibiting Protein kinase A (PKA) activation of extracellular signal-regulated kinase (Son, Ubuka, Millar, Kanasaki, & Tsutsui, 2012). In Leydig cells of pigs, RFRP3 may similarly function to block LH-induced PKA activation of steroidogenic enzymes.

The role of the RFRP system in boars may not be limited to steroidogenesis. Avian *GnIH* mRNA was present in the germ cells of quail (Bentley et al., 2008). In Syrian hamsters, NPFFR1 protein was observed in myoid cells of the seminiferous tubules as well as in spermatocytes, round spermatids, and elongating spermatids (Zhao et al., 2010). Protein for NPFFR2 was only found in elongating spermatids, but mRNA and precursor protein of the *NPVF* gene (RFRP precursor) were found in spermatocytes and spermatids (Zhao et al., 2010). In the testes of boars, RFRP precursor protein was also observed in the seminiferous tubules (Zheng et al., 2015). These data indicate the potential for RFRP to contribute to germ cell differentiation and spermatogenesis in boars.

3 | GONADOTROPIN-RELEASING HORMONE II (GNRH-II) AND ITS RECEPTOR (GNRHR-II)

In addition to the classical GnRH-I, a second GnRH isoform, GnRH-II, (His^5,Trp^7,Tyr^8) has been identified in vertebrates (Fernald & White, 1999). In fact, GnRH-II has been identified in every vertebrate class, representing 500 million years of evolution, which suggests that GnRH-II is the most ancient isoform of GnRH (Millar et al., 2004). The structure of GnRH-II has remained completely conserved among vertebrates, indicating high selective pressure to maintain its structure and suggesting that it has a critical function (Millar & King, 1987). Unlike GnRH-I, GnRH-II is ubiquitously expressed, with transcript levels highest in extra-hypothalamic tissues (White, Eisen, Kasten, & Fernald, 1998). Little GnRH-II protein was identified in hypothalamic regions known to regulate gonadotropin secretion in the musk shrew (Rissman, Alones, Craig-Veit, & Millam, 1995), and we likewise detected only low levels of GnRH-II in the hypothalamus of the boar (Desaulniers et al., 2015). These data suggest that GnRH-II has a distinct role from GnRH-I in boars.

A receptor specific to GnRH-II (GnRHR-II) has also been discovered in mammals (Millar et al., 2001; Neill, Duck, Sellers, & Musgrove, 2001), although it was originally cloned in the African catfish (Tensen et al., 1997). GnRHR-II is a 7-transmembrane G-protein-coupled receptor consisting of 379 amino acids (Tensen et al., 1997) and an intracytoplasmic tail. GnRHR-II possesses only 40% sequence identity to GnRHR-I (Neill et al., 2001). Gene coding errors (e.g., frameshift mutations, premature stop codons) prevent the production of a functional GnRHR-II in many species (Millar, 2003); however, the appropriate gene sequence for a fully functional receptor is produced in the pig (Stewart, Katz, Millar, & Morgan, 2009). Porcine GnRHR-II has 90% sequence identity to its ortholog in African green monkeys (Neill, Duck, & Musgrove, 2002a, 2004). Production of inositol triphosphate following GnRH-II treatment of COS-7 cells overexpressing porcine GNRHR-II indicates that this receptor is functional (Neill et al., 2002b), and has a preference for GnRH-II, given the half-maximal response concentration (EC_{50}) of 0.5 nM for GnRH-II compared to 220 nM for GnRH-I (Neill et al., 2002b). Like its ligand, the gene encoding GnRHR-II (*GNRHR2*) is ubiquitously expressed, with transcript levels highest in the testis (Millar et al., 2001), suggesting its important role in testis biology.

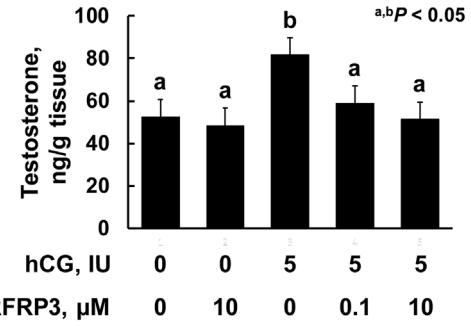

FIGURE 1 Secretion of testosterone from explant cultures of mature boar testes. Explants were cultured in the absence or presence of RFamide-related peptide 3 (RFRP3) for 120 min; human chorionic gonadotropin (hCG) was included as a positive control. Treatments were replicated in independent experiments ($n = 5$). Data show LSmeans ± standard errors. [a,b]Means without a common superscript are different ($p < 0.05$)

3.1 | GnRH-II and gonadotropin secretion

GnRH-II was initially hypothesized to function, like GnRH-I, as a stimulator of gonadotropin release. Administration of GnRH-II increased plasma LH concentrations during the follicular phase in rhesus monkeys (Lescheid et al., 1997) and stimulated ovulation in female musk shrews—albeit with only about 10% the efficacy of GnRH-I (Rissman et al., 1995). Release of LH from cultures of mammalian pituitary cells increased after treatment with GnRH-II, but with only 2% of the potency of GnRH-I (Millar & King, 1983; Millar, Milton, Follett, & King, 1986). These data indicated that, compared to GnRH-I, GnRH-II is a poor stimulator of gonadotropin secretion; additional data subsequently indicated that GnRH-II and its receptor are not major physiological stimulators of gonadotropin release in mammals (Schneider & Rissman, 2008). GnRH-II was instead reported to bind and activate GnRHR-I (Millar et al., 2001), and later experiments with sheep (Gault, Maudsley, & Lincoln, 2003), musk

shrews (Kauffman, Wills, Millar, & Rissman, 2005), and primates (Densmore & Urbanski, 2003; Okada, Murota-Kawano, Kakar, & Winters, 2003), concluded that GnRH-II weakly stimulates secretion of LH through GnRHR-I. Pretreatment with a GnRHR-I antagonist completely attenuated the effect of GnRH-II on gonadotropin secretion and ovulation (Densmore & Urbanski, 2003; Kauffman et al., 2005; Okada et al., 2003). Moreover, GnRH-II-induced secretion of LH from primary cultures of porcine pituitary cells was blocked by pre-treatment with a GnRHR-I antagonist (Neill et al., 2002a). We observed a minimal increase in secretion of LH in GnRH-II-treated boars compared to boars treated with GnRH-I (Desaulniers et al., 2015). Likewise, immunization of boars against GnRH-II did not affect gonadotropin secretion (Bowen et al., 2006). Nevertheless, a role for GnRH-II and its receptor to directly regulate the testis cannot as yet be ruled out.

3.2 | GnRH-II and paracrine regulation of testis function

We and others found that GnRH-II and its receptor are present in the testes (Desaulniers et al., 2015; Lin, Liu, Poon, Leu, & Huang, 2008; Millar et al., 2001; Neill et al., 2001; van Biljon et al., 2002; White et al., 1998). GnRH-II protein resides within the seminiferous tubules of the boar, particularly in germ cells and Sertoli cells (Desaulniers et al., 2015). We detected GnRHR-II on mature spermatozoa (Desaulniers, Cederberg, Mills, Lents, & White, 2015), developing germ cells, and the plasma membrane of porcine Leydig cell (Desaulniers et al., 2015). The abundance of GnRH-II protein in the testis was sevenfold greater than within the anterior pituitary gland or hypothalamus, and GnRHR-II protein was sixfold greater in the testis than in the anterior pituitary gland (Desaulniers et al., 2015). The abundance and localization of GnRH-II and its receptor in the porcine testis provide strong supportive evidence of an autocrine/paracrine role for GnRH-II in steroidogenesis and/or spermatogenesis in the boar.

The abundance of *GnRHR-II* mRNA increased 76-fold in the testes of post-pubertal compared to pre-pubertal boars (Voss, 2013). Transcription of *NPVF* gene (RFRP precursor), *NPFFR1*, and *NPFFR2* in the boar testis decreased during this same period (Zheng et al., 2015). In mice, testicular expression of *GnRH-I* and the *Npvf* gene (RFRP precursor) appear to coordinate steroidogenic activity during pubertal development (Anjum, Krishna, Sridaran, & Tsutsui, 2012). RFRP3 and NPFF receptors may function similarly in the boar, controlling the pubertal timing of increased testicular steroidogenesis—although we expect this involves GnRHR-II in boars.

Previous evidence in boars implicates GnRH-II in paracrine regulation of testosterone secretion. When boars were treated with a GnRHR antagonist (SB-75), hCG-induced secretion of testosterone was blunted (Wise, Zanella, Lunstra, & Ford, 2000), suggesting direct action of GnRHR-I in the testis. In agreement, Zanella and colleagues also reported that SB-75 attenuated the hCG-induced release of testosterone from cultured testicular explants of boars(Zanella, Lunstra, Wise, Kinder, & Ford, 2000). Moreover, concentrations of testosterone in boars treated with SB-75 remained suppressed even after concentrations of LH returned to normal (Zanella, Lunstra, Wise, Kinder, & Ford, 2000). Thus, a testicular GnRH receptor appears to promote steroidogenesis in the boar; however, expression of the gene encoding GnRHR-I (*GNRHR1*) has not been detected in the porcine testis. Furthermore, SB-75 can inhibit activation of GnRHR-II (Maiti et al., 2003). When boars were immunized against GnRH-II, concentrations of testosterone were reduced even though LH concentrations were unchanged (Bowen et al., 2006). We observed that GnRHR-II protein was indeed present within the interstitial compartment of the boar testis (Desaulniers et al., 2015), indicating that testicular GnRHR-II could directly modulate steroidogenesis.

Based on these data, we hypothesized that locally produced GnRH-II binds to the GnRHR-II on porcine Leydig cells to stimulate testosterone secretion. We tested this hypothesis by first examining if GnRH-II could stimulate testosterone secretion ex vivo. Testicular explants from boars were cultured in the presence of GnRH-II, hCG, or vehicle control. Both hCG and GnRH-II treatments stimulated a similar release of testosterone (Desaulniers et al., 2015). These data demonstrated, for the first time, that GnRH-II has a direct effect on testosterone secretion from the testis of boars. We then examined the effect of GnRH-II in vivo by collecting serial blood samples from boars treated with both isoforms of GnRH. Both the GnRH-I and -II treatments increased serum concentrations of testosterone in boars. The GnRH-I treatment, however, robustly stimulated secretion of LH

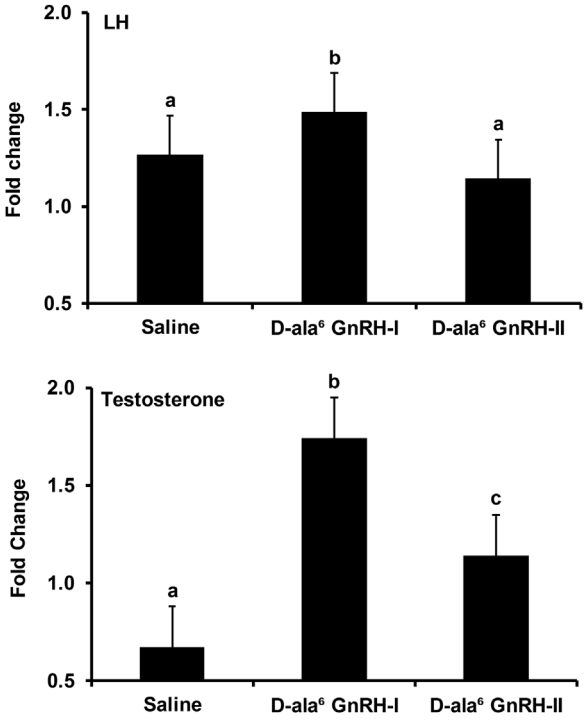

FIGURE 2 Secretion of LH and testosterone in mature boars after intratesticular treatment with saline, GnRH-I (D-ala^6 GnRH-I), or GnRH-II (D-ala^6 GnRH-II). The LSmeans ± standard errors are expressed as the fold change over an average of the pretreatment means for each treatment. ^{a,b,c}Means without a common superscript are different ($p < 0.01$)

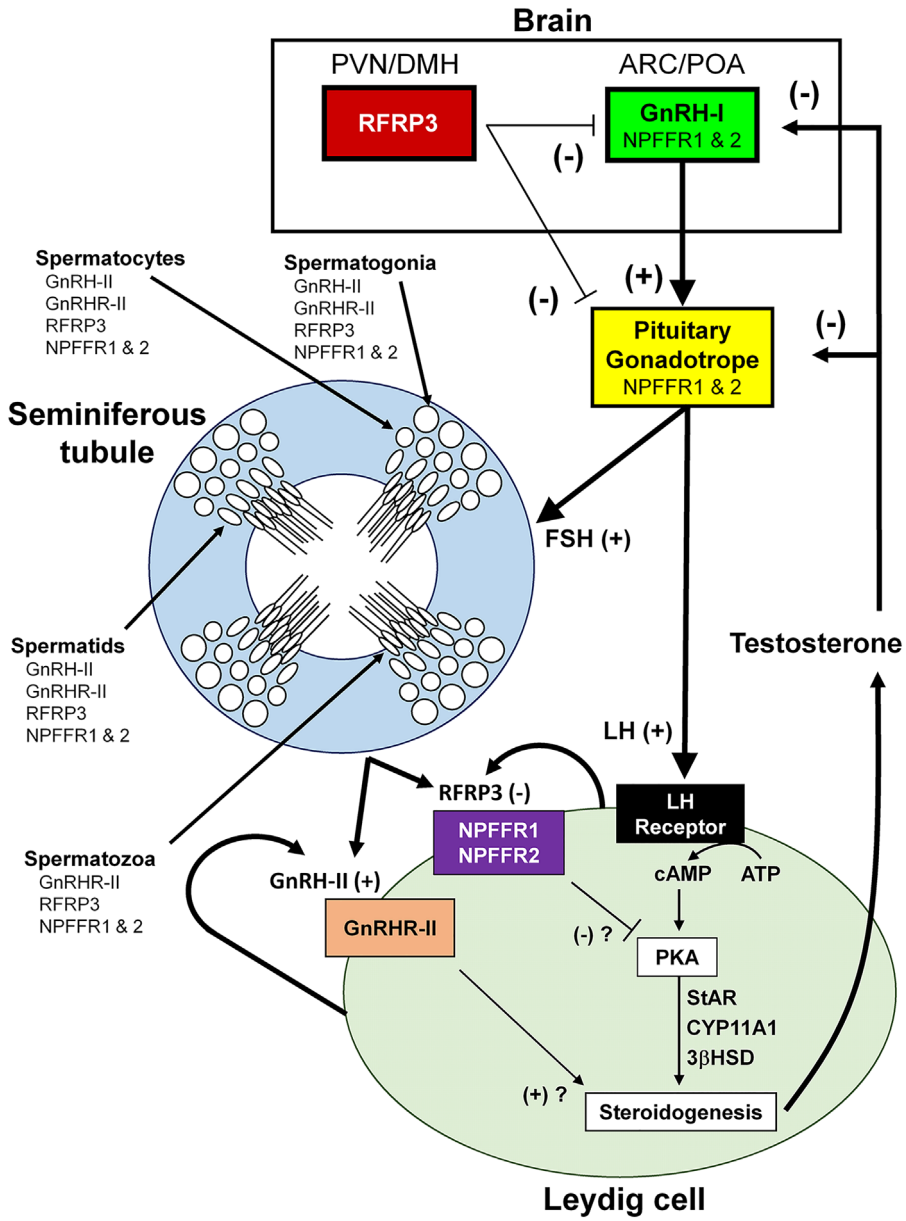

FIGURE 3 A model for the regulation of testicular function in the boar by RFRP3 and GnRH-II. The classic mechanism regulating testicular function in boars involves GnRH-I-stimulated secretion of the gonadotropins LH and FSH. Testosterone secretion is stimulated by LH, which triggers PKA to activate enzymes involved in steroidogenesis. Spermatogenesis is stimulated by FSH action on Sertoli cells in the seminiferous tubules. Testosterone feedback to the hypothalamus and adenohypophysis negatively regulates gonadotropin secretion. Neurons in the paraventricular nucleus (PVN) and dorsomedial nucleus of the hypothalamus (DMH) contain RFRP3 neurons that project fibers to the preoptic area (POA) and median eminence (ME) to control secretion of LH. Both GnRH-I neurons in the POA and pituitary gonadotropes express RFRP3 receptors (neuropeptide FF receptor [NPFFR] 1 and 2). RFRP3 inhibits gonadotropin secretion by suppressing GnRH-I neurons or gonadotrope cells directly. Regulation of the hypothalamic-pituitary axis by RFRP does not appear to be prominent in boars. Instead, RFRP3 and GnRH-II are expressed in germ cells and interstitial cells of the testes. Leydig cells of boars express receptors for GnRH-II and RFRP3, indicating they may act in an autocrine or paracrine fashion. Secretion of testosterone from boar testes is inhibited by RFRP3, potentially by preventing PKA activation of enzymes associated with steroidogenesis. In contrast, GnRH-II stimulates testosterone secretion in the boar testis—although the mechanism remains to be elucidated. Expression of receptors for RFRP3 and GnRH-II in germ cells of boars suggests a possible role for them in the regulation of spermatogenesis and fertility. cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate

whereas GnRH-II treatment had a minimal effect on LH secretion (Desaulniers et al., 2015). These data support our hypothesis that GnRH-II is eliciting testosterone secretion directly at the testis rather than indirectly by stimulating LH secretion.

We then examined the effect of intratesticular injection of GnRH isoforms on concentrations of testosterone in boars, thereby avoiding stimulation of the anterior pituitary gland. Serial blood samples were collected from boars before and after treatment with GnRH-I, GnRH-II,

or saline. Concentrations of LH and testosterone were increased ($p < 0.01$) in boars treated with GnRH-I compared with saline-treated boars (Figure 2). Concentrations of testosterone were also increased ($p < 0.01$) in boars treated with GnRH-II compared to saline-treated boars, but LH remained unaffected (Figure 2). Overall these data confirm that GnRH-II and its receptor can exhibit paracrine action to regulate testosterone secretion in boars.

4 | CONCLUSIONS

Testosterone secretion in the boar is negatively regulated by RFRP3 and positively regulated by GnRH-II. Although RFRP3 and GnRH-II can regulate in vitro secretion of LH from porcine pituitary cells, their effects on in vivo secretion of LH in pigs is minimal. The effect of these peptide hormones on testosterone secretion in the boar does not appear to involve the hypothalamic–pituitary axis (Figure 3). Receptors for RFRP3 and GnRH-II are present in the testes of boars. A direct effect of RFRP3 on the inhibition of testosterone release in testicular explant and Leydig cell cultures from boars involves inhibiting transcription of steroidogenic enzymes. Conversely, GnRH-II directly stimulates secretion of testosterone from boar testes both in vitro and in vivo, although how this occurs remains to be elucidated. Expression of RFRP3 receptors in the testes of boars are reduced, whereas expression of GnRH-II receptor is dramatically up-regulated, during the onset of puberty. This reciprocal pattern of expression may represent a complex mechanism that coordinates steroidogenesis in the developing testis of the boar. Further study into how RFRPs and GnRH-II interact during development will be important in order to provide clues to the pubertal transition of testosterone secretion in the boar. A greater understanding of the autocrine–paracrine mechanisms regulating testosterone secretion in the boar testis is also needed to unravel how to improve boar development, which should lead to methods that increase semen production and quality.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

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